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(54) **METHOD FOR THE DETECTION OF CHLAMYDIA TRACHOMATIS AND A KIT FOR IT**
 VERFAHREN FÜR DEN NACHWEIS VON CHLAMYDIA TRACHOMATIS UND KIT DAFÜR
 PROCEDE ET TROUSSE POUR LA DETECTION DE CHLAMYDIA TRACHOMATIS

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Description

[0001] The present invention relates to a method for the detection of *Chlamydia trachomatis* and to a kit for it.

[0002] *Chlamydia trachomatis* is one of the non-gonococcal urethritis pathogens that contains a cryptic plasmid [M. Commanducci et al., Mol. Microbiol., 2, No. 4 (1998), pp. 531-538]. A method for detecting *Chlamydia trachomatis*, a method for amplifying a partial sequence of the cryptic plasmid by a gene amplification process is known (JP 2719225 and JP 3127135).

[0003] An amplification assay for *Chlamydia Trachomatis* has been described, using thermal strand displacement (EP 915170). A fluorescence based detection assay using a detector probe has been described (EP915173). An assay for detection of *Chlamydia Trachomatis* infection has been described, based on polypeptides comprising an *Chlamydia Trachomatis* antigen (WO 01-4074). A method for performing a PCR detecting *Chlamydia Trachomatis* has been described (EP 1598431). A DNA amplification method and kit therefore, based on PCR amplification technology has been described (EP 1602734).

[0004] It has been found that problems arise with the observation and/or detection of *Chlamydia trachomatis* under certain conditions. An article by Söderblom et al. (Euro Surveill. 2006 Dec. 7; 11 (12)): E061207.1) entitled "Impact of a genetic variant of *Chlamydia trachomatis* on national detection rates in Sweden" reports that part of the sexually transmitted *Chlamydia trachomatis* infections could not be observed in Sweden by the standard laboratory tests produced by Abbott and Roche, and that *Chlamydia* bacteria with a variation in the genetic region to which the primers had been directed were not observed.

[0005] There is therefore a need for alternatives to the standard tests whereby for example such genetic variants of *Chlamydia trachomatis* can in fact be observed.

[0006] The goal of the present invention is therefore to provide a method for the rapid detection of *Chlamydia trachomatis*, which has a good sensitivity and specificity, and a kit for it.

[0007] The goal of the invention is achieved by a method for the detection of *Chlamydia trachomatis*, which method comprises the performance of a DNA amplification involving the use of a primer pair by employing DNA derived from a sample as a template, and the detection of an amplification product, characterized in that the primer pair employed for the DNA amplification is designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 4261 to 4320 and 4351 to 4391 in the nucleotide sequence of SEQ ID No. 1 (i.e. they are in the ORF3 region of the above-mentioned cryptic plasmid, as is well known to the person skilled in the art).

[0008] The inventors have found that when a DNA amplification, such as for example PCR (whenever PCR is mentioned in the context of the present Application, the intended meaning is DNA amplification, of which PCR is an embodiment) is carried out with primers that are designed on the basis of such specific regions in the cryptic plasmid of *Chlamydia trachomatis*, *Chlamydia trachomatis* can be rapidly observed, and for example also the known *Chlamydia trachomatis* variants, such as those found for example in Sweden can also be observed. Another suitable method of DNA amplification for the determination of pathogens is described in WO 2005/060725 and is hereby incorporated by reference.

[0009] The primer pair for the DNA amplification, such as PCR, is preferably designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 4261 to 4320 and 4351 to 4391, more preferably to the nucleotide numbers 4291 to 4320 and 4355-4385 and most preferably to the nucleotide numbers 4296 to 4318 and 4361 to 4382 of SEQ ID No. 1.

[0010] It has been found that the results improve as the preference increases for the nucleotide regions on the basis of which the separate primers for the primer pair are designed. In other words, the preference given above is based on the better results obtained with the primer pair in the preferred regions specified.

[0011] In other words, the primer pair with the greatest preference is designed on the basis of nucleotide sequences of the regions in SEQ ID No. 1: GGATT GACTCCGACA ACGTATTC and TGCCCTTCT AATGGCAATGAT, corresponding respectively to the region of 4296 to 4318 and of 4361 and 4382 in SEQ ID No. 1. A first primer is preferably directed to the whole region GGATT GACTCCGACA ACGTATTC, and the second primer of the primer pair is directed to the whole region TGCCCTTCT AATGGCAATGAT. The person skilled in the art will understand that the other regions described above can be established on the basis of what has been said above.

[0012] The person skilled in the art will understand that DNA derived from a sample means DNA that is isolated or is obtained from an organism, such as a person, to be tested, but it also covers copies, replicates and processed forms of such DNA, obtained for example by chemical synthesis or PCR, starting with the original DNA isolated from the organism.

[0013] The sample used is not particularly subject to restrictions, provided that it contains or can contain *Chlamydia trachomatis*. Examples of this comprise urine, urethral (urinary tube) scrapings, cervical smears etc. DNA can be extracted from these samples by the method known to the person skilled in the art, whereby DNA, including DNA of the cryptic plasmid of *Chlamydia trachomatis*, can be prepared. It is particularly preferable to employ the Roche COBAS CT DNA preparation kit, because it has been found to give particularly advantageous results. It will be obvious to the person skilled in the art that the DNA can be either isolated pure DNA or DNA that is present in an unprocessed lysate.

[0014] The primer pairs that can be employed in the invention can therefore be chosen in the regions described above and in particular in such a way that the nucleotide region can be amplified/replicated between the two regions.

[0015] The primer pair used in the present invention is designed on the basis of a nucleotide sequence of the region corresponding to the nucleotide numbers 4261 to 4320, preferably 4291 to 4320 and more preferably 4296 to 4318 (first region) and 4351 to 4391, preferably 4355-4385 and more preferably 4361 to 4382 (second region), as mentioned above and in SEQ ID No. 1, so that the nucleotide sequence can be amplified (replicated) between these two regions with the aid of the primer pair.

[0016] According to the invention, the length of the primers is for example between 10 and 40 nucleotides. Furthermore, the position of each region and the length of the primers are preferably chosen so that the T_m value of the primer in question and the corresponding template DNA lies between 50 and 70°C, and so the annealing temperature used in the PCR can be set at a relatively high value. The T_m value used here is a value that is calculated by the nearest neighbour base pair analysis. The primers can basically have the same T_m value.

[0017] The person skilled in the art will understand that the term "designed on the basis of" means that, with the stipulations specified for the primer, such as the T_m value and the length of the primer, which are described herein, the primer is designed as such that it can be complementary to the sequence in either the sense strand or the antisense strand of the nucleotide sequence of the regions according to the invention described above and especially in the claims. The design therefore starts with the sequence on which the primer has to bind, and with the stipulations and in the context of the present invention, the primer can be complementary to the sequence on which it has to bind.

[0018] PCR is the method of detection according to the present invention, and it can be carried out in accordance with the normal PCR method, provided that the DNA obtained from the sample is used as a template, and that a specific primer set according to the invention is used. In particular, real-time PCR is employed in the method according to the invention (TaqMan assay, see the Example), because this has given particularly good results.

[0019] The primer pair can be designed in such a way that the nucleotide sequence between the two regions (i.e. the region between the location where a first primer will bind under the PCR conditions and the location where a second primer will bind under the PCR conditions) can be replicated (amplification). This means that one primer can be a sense primer, and the other can be an anti-sense primer.

[0020] The nucleotide sequence reproduced in SEQ ID No. 1 is derived from the cryptic plasmid of *Chlamydia trachomatis* (L2 strain), deposited with the GeneBank under access number X07547, based on Commanducci's publication (see above), and reproduced in Dutch Patent Application No. 103345, which is the priority document for the present Application and which is included here by reference. The person skilled in the art will understand that a PCR carried out with a primer pair according to the invention will be able to lead to the replication of the DNA in this cyclic plasmid. Since the cryptic plasmid is a cyclic plasmid, it has two nucleotide sequences between the two regions on the basis of which the primer pair is designed. However, the primers are usually so designed that the shorter nucleotide sequence can be replicated (amplification).

[0021] The examples of the preferred primer pairs according to the present invention comprise a combination of primer pairs that are designed on the basis of a nucleotide sequence of a region corresponding to the nucleotide numbers 3654 to 4320, preferably 4261 to 4320, more preferably 4291 to 4320 and most preferably 4296 to 4318 (first region) and on the basis of a nucleotide sequence of a region corresponding to the nucleotide numbers 4355-4385 and preferably 4361 to 4382 (second region).

[0022] Another preferred example of the primer pairs according to the present invention involves a combination of primer pairs designed on the basis of a nucleotide sequence of a region corresponding to the nucleotide numbers 4291 to 4320, preferably 4296 to 4318 (first region) and a nucleotide sequence of a region corresponding to the nucleotide numbers 4351 to 4391, preferably 4355 to 4385 more preferably 4361 to 4382 (second region).

[0023] Designing the nucleotide sequence of the primer pair is obvious to the person skilled in the art if he knows the corresponding sequences in SEQ ID No. 1, disclosed in this invention. SEQ ID No. 1 is one of the DNA strands of the double-stranded cryptic plasmid. This strand is called here the sense strand. The opposite strand, which is complementary to this sense strand, is called here the anti-sense strand. As for the amplification with the aid of for example PCR, the person skilled in the art will know that he should base the sequence of the primer of the primer pair to be used on the sequence described above, which corresponds to the sequence of the sense strand of the cryptic plasmid (the so-called "forward" primer). He will base the sequence of the other primer of the primer pair on the sequence of the anti-sense strand, called the "reverse" primer.

[0024] The sequences of the primers in question are preferably so chosen that the completed amplification leads to an amplification product that is specific to the envisaged cryptic plasmid's sequence to be amplified. The sequence of the selected primers therefore need not necessarily be completely identical to the said sequence in SEQ ID No. 1 described above (nor does it need not be fully complementary to it). Mismatches are admissible, provided that the specificity of the amplification is retained. By preference, the sequence of the primers agrees completely with the one defined by SEQ ID No. 1 (or with the sequence that is complementary to it).

[0025] In those cases occurring in practice where the specificity is relevant, for example when polymorphism can be

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expected in the DNA to be amplified, especially on sites in the DNA where one of the primers to be used should hybridize, it is preferable to use more than one primer whose sequence is complementary to the various polymorphisms. In such a primer set given below, there are two forward primers (which differ from each other only in one site of the sequence) and a single reverse primer. This makes it possible to detect a polymorphism in *Chlamydia trachomatis* G4307A in SEQ ID No. 1, which position is occupied by one of the primers to be used according to the invention. It is of course also possible in such a case to use a single forward primer and more than one reverse primer. If one wants to demonstrate a number of polymorphisms in a region where a primer hybridizes, one can employ the same number of different primers, each being specific to one such polymorphism.

[0026] The primer pair for the above-mentioned specific region can be designed with the aid of the methods that are known to the person skilled in the art, taking the PCR conditions into account. The primer pair can be designed by using a computer program.

[0027] The primer pair can be designed as a mixed primer obtained by mixing two or more primers for the sense or the anti-sense primer, or for both of them. When a nucleotide mutation occurs in the region for which the primer has been designed, the sensitivity of the observation/detection can be increased by using a mixed primer (see also below).

[0028] Although the PCR conditions can be determined in accordance with the customary PCR method, the annealing temperature can be set at a relatively high value, owing to the specific primer pairs mentioned here. The annealing temperature is usually between 50 and 70°C.

[0029] Another aspect of the invention relates to a kit for the detection of *Chlamydia trachomatis* by performing a PCR in which the DNA obtained from a sample is used as a template.

[0030] The kit that is particularly envisaged here for the detection of *Chlamydia trachomatis* by a PCR, using the DNA obtained from a sample as a template comprises a primer pair designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 4261 to 4320 and 4351 to 4391 in the nucleotide sequence in SEQ ID No. 1.

[0031] More in particular, good results were obtained with a kit according to the invention that is characterized in that the primer pair is designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 4261 to 4320 and 4351 to 4391; preferably to the nucleotide numbers 4291 to 4320 and 4355-4385, and most preferably to the nucleotide numbers 4296 to 4318 and 4361 to 4382 of SEQ ID No. 1.

[0032] 10 Particularly favourable results are obtained with a kit that is characterized in that the primer pair in the kit is designed on the basis of nucleotide sequences of the regions in SEQ ID No. 1: GGATT GACTCCGACA ACGTATTC (SEQ ID No. 2) and TGCCCTTTCT AATGGCAATGAT (SEQ ID No. 3). As already explained above, the above sequences of the primers are all reproduced on the basis of the sense strand of the cryptic plasmid.

[0033] 15 Another aspect of the invention relates to a primer pair designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 3654 to 4320 and 4351 to 4448 in the nucleotide sequence of SEQ ID No. 1, in agreement with the goal of the invention, such as for example the detection of *Chlamydia trachomatis*. This means that the 20 primer pairs envisaged according to the invention are those which are suitable for use for the detection of *Chlamydia trachomatis*.

[0034] More especially, the primer pairs according to the invention are characterized in that they are designed on the basis of the nucleotide sequences of the regions corresponding to the 25 nucleotide numbers 4261 to 4320 and 4351 to 4391, preferably to the nucleotide numbers 4291 to 4320 and 4355-4385, and most preferably to the nucleotide number 4296 to 4318 and 4361 to 4382 of SEQ ID No. 1.

[0035] In the most preferred case, the primer pair is designed on the basis of nucleotide sequences 30 of the regions in SEQ ID No. 1: GGATT GACTCCGACA ACGTATTC (SEQ ID No. 2) and TGCCCTTTCT AATGGCAATGAT (SEQ ID No. 3).

[0036] In a preferred embodiment, a primer pair with the following sequences is used:

CT forward1 5'-GGA TTG ACT CCG ACA ACG TAT TC-3'
CT reverse 5'-ATC ATT GCC ATT AGA AAG GGC A-3'

[0037] As regards its sequence, CT forward corresponds to the nucleotides 4296-4318 of SEQ ID No. 1, and CT reverse corresponds to the anti-sense sequence of nucleotides 4361-4382 of SEQ ID No. 1.

[0038] In an advantageous embodiment of the invention, a primer set is also provided that comprises a primer pair according to the invention, together with a supplementary primer that differs in just one nucleotide from one of the primers of the primer pair as regards its sequence. As already explained above, this offers the possibility of demonstrating one or more polymorphisms in the regions in the cryptic plasmid with which one of the primers of the primer pair hybridizes.

[0039] It is even more preferable to add also a second forward primer to the primer pair in order to make it possible to detect G4307A polymorphism:

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CT forward2 5'-GGA TTG ACT CCA ACA ACG TAT TC-3'

5 [0040] Another aspect of the invention relates to a hybridization probe that can hybridize in the region replicated with the aid of the primer pair according to the invention. The hybridization probe preferably contains an oligonucleotide that is designed on the basis of the nucleotide sequence of the region corresponding to the nucleotide numbers 4320 to 4355, the hybridization probe being preferably designed on the basis of the nucleotide sequence TACGTGTAGG CG-GTTTAGAA AGCGGGTGT (SEQ ID No. 4). In the most preferred case, the hybridization probe is aimed at the whole
10 sequence described above and gives exceptionally good detection results. In particular, the hybridization probe has the following sequence:

CT reverse 5'-ACA CCG CTT TCT AAA CCG CCT ACA CGT AA-3'

15 [0041] Such a hybridization probe is generally provided with a label for easy detection. The person skilled in the art knows the conditions under which hybridization is used; these conditions may be for example the same as those described in US 2006/0246447.

[0042] The person skilled in the art will understand that the hybridization probe used for the detection can be complementary either to a sense strand or to an anti-sense strand.

20 [0043] The chain length of the oligonucleotide is normally 15 to 30 nucleotides, and its T_m value can be 50 to 70°C.

[0044] When real-time PCR is used, the hybridization probe preferably has a T_m value that is 1 to 5 degC higher than the T_m value of a primer, so that the probe can hybridize with the target sequence before the primer is hybridized.

[0045] The invention is explained in more detail with the aid of the following examples and drawings, where:

25 Fig. 1 is a graph that shows the limit of detection of *Chlamydia trachomatis* and the internal reference (IR) sample in the real-time PCR test. The DNA used was derived from serial dilutions of *Chlamydia trachomatis* with 100, 10, 1, 0.1, e, 0.001 IFU/PCR. The slope of the graph is -2.9, the intercept is 29.4 and R^2 is 0.994. The x axis of the graph represents the initial IFU values of *Chlamydia trachomatis*, expressed as log IFU equivalents per PCR reaction. The y axis represents the threshold cycle (C_T).

30 Fig. 2 is a graph for: A) DNA isolated from pure *Chlamydia trachomatis* (CT), B) *Chlamydia trachomatis* mixed with other bacteria, and C) mixed DNA that contains no *Chlamydia trachomatis* DNA, all done in duplicate. The x axis of the graph represents the number of cycles (cycle number) and the y axis represents the fluorescent intensity after the elimination of the background signal.

35 Examples

Example 1

40 Introduction

[0046] *Chlamydia trachomatis* is an obligate intracellular Gram-negative bacterium. Fifteen different serotypes of it are known at present, including eight (D-K) that cause urogenital infections. The infection is generally asymptomatic in women, and untreated infections can lead to endometritis, salpingitis and infertility. It is believed that screening for asymptomatic infections would reduce the transmission of *CT* and the development of serious complications¹ (raised numbers denote literature references listed later). Nucleic acid replication tests have now replaced antigen detection and cell culture for the diagnosis of infections by *CT*. A great number of commercial and in-house nucleic acid replication tests have been described. Most of the earlier replication tests²⁻⁸ have major drawbacks, such as manual processing, open systems, separate steps for replication and Amplicon analysis, and sensitivity to contamination. It has been found
50 that real-time PCR is a sensitive alternative that is easy to carry out. Despite improvements in real-time PCR, the type-specific inhibitory substances present in some clinical samples cannot always be reliably removed from the sample during preparation. This inhibition can be recognized by the use of an internal reference. Especially when samples that are not approved by the FDA, such as rectal smears, are to be tested for the presence of *trachomatis*, a type of sample that has received much attention with the recent outbreak of *Chlamydia trachomatis*-induced LGV amongst men who have sex with men (MSM). This study deals with the development and validation of real-time PCR with an internal
55 reference that takes care both of inhibition and nucleic acid extraction from clinical samples.

Material and methodsDevelopment of real-time PCR

5 [0047] We have developed a real-time PCR (TaqMan assay) directed at the cryptic plasmid of *trachomatis*. The primers and the probe were developed with Primer Express 1.0 (from Opliet Biedsysteem). Real-time PCR reactions were carried out in a PCR volume of 30 μ l, containing 1x TaqMan Mastermix (from Applied Biosystems), 300 nM of each primer, 150 nM of each probe, and 10 μ l of the extracted DNA, apart from the samples that were prepared with the Roche Kit (14 μ l). Replication and detection were carried out with an ABI Prism 7000 sequence detection system (from Applied Bio-
10 systems) under standard PCR conditions, stipulated by the manufacturer, using 45 cycles.

Determination of the sensitivity

15 [0048] The sensitivity was determined by carrying out the real-time PCR on a previously described serial dilution of DNA from the LGV L2 strain of *Chlamydia trachomatis* (region 10-0.001 IFU). The results were compared with our previously reported in-house CT-PCR and a commercially available PCR (COBAS Amplior from Roche).

Determination of the specificity

20 [0049] The specificity was determined in silico, by carrying out a BLAST search for all the oligonucleotides used in this study. The specificity was further investigated by carrying out a CT-PCR on various bacterial species, namely *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 1228), *Klebsiella pneumoniae* (ATCC 13883), *Candida albicans* (ATCC 90028), *Protheus mirabilis* (ATCC 43071), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecium* (clinical isolate), *Moraxella catarrhalis* (clinical isolate), *Haemophilus influenza* (clinical isolate),
25 as well as a panel containing all the serotypes of *Chlamydia trachomatis*.

Evaluation of real-time PCR

30 [0050] Clinical samples (cervical smears) that had been positive (N = 100) and negative (N = 100) in the COBAS Roche PCR method were tested in the new real-time PCR after 1) the isolation of DNA from the original samples collected in 2 SP with the HPPTP Kit (from Roche), and 2) the commercially available COBAS CT DNA Preparation Kit (CDPK).

Validation of the internal reference

35 [0051] The internal reference used in this study was an inventive and particularly suitable reference sample that makes it possible to check the tests for good performance. It uses the same primer binding sites as the new real-time CT assay, but has an artificial sequence as the probe binding site for monitoring the DNA isolation, including the lysis of the samples. In other words, the internal reference is a target DNA sequence that can be observed by using the same primers as those which are used for observing the *Chlamydia* described here, but it can also be observed with a hybridization probe
40 that is different from the one used for observing the *Chlamydia* described here. For example, since the DNA at which this different hybridization probe is directed is different from the one used for the detection of *Chlamydia*. The target of the internal reference was transferred into the genome of an *E.coli* (DH5 α strain) with the aid of the suicide vector pBSL182. By spiking the altered *E.coli* DH5 α strain in the clinical samples, the DNA isolation and the primer-specific inhibition is monitored in each PCR amplification. The exact amount of spiked *E.coli* DH5 α was determined by using a
45 serial dilution (0-20,000 cfu). The spiking was carried out both on the COBAS negative and on the COBAS positive clinical cervical smears, prior to the isolation of DNA in order to determine the amount of the internal reference sample needed for a reliable recovery without a drop in the sensitivity of the *Chlamydia trachomatis* assay. DNA was isolated from all the samples as described above, either by HPPTP or by CDPK.

Internal reference spiking of clinical samples

50 [0052] The clinical samples mentioned above were spiked with the optimum quantity of IR in order to validate the internal reference, Which is also used to check the sample preparation procedure.

55

Results

Sensitivity and specificity of the new real-time PCR

5 [0053] The lower limit of detection was determined by using serial dilutions of a *Chlamydia trachomatis* LGV L2 strain and analysing the results by real-time PCR, as described before⁶. The sensitivity was found to be 0.01 IFU/PCR. This sensitivity is identical to that of the previously developed non-real-time PCR, while a sensitivity of 1 IFU was reported for the commercially available PCR of Roche and for LCr of Abbott. The assay was capable of detecting all the serotypes of *Chlamydia trachomatis* present in the panel. The specificity of the real-time CT assay was investigated by comparing the quantifications of pure *Chlamydia trachomatis* with that of DNA from *Chlamydia trachomatis* mixed with DNA isolated from nine other bacterial types. As shown in Fig. 2, the PCR did not show any cross-reaction with alien DNA.

Evaluation of real-time PCR with clinical samples

15 [0054] All the clinical samples that had been found to be either positive (N = 100) or negative (N = 100) in the COBAS Roche PCR were tested by the new real-time PCR method after the isolation of DNA either by HPPTP or by the CDPK procedure: all the samples gave the same results as the original COBAS method.

Validation of the internal reference

20 [0055] Prior to the preparation of the samples with the aid of the HPPTP kit or the CDPK procedure, ten clinical samples were spiked with various serial dilutions of the modified *E.coli* DH5 α strain, which showed that the best amount of the internal reference sample was 200 cfu in the HPPTP procedure and 2000 cfu in the CDPK procedure. To determine whether the addition of IR results in the same sensitivity, the same serial dilutions were tested after the addition of 200 cfu of IR. As shown in Fig. 1, the assay was linear between 1000 and 0.01 IFU/PCR for the assay including the IR, which means that 0.01 IFU/PCR remained the lower limit of detection.

Validation of the internal reference in clinical samples

30 [0056] Samples that were negative for *Chlamydia trachomatis* and contained the internal reference sample were replicated, and the resulting C_1 values were averaged to find the analytical variation in the values for *Chlamydia* and for the inhibition cut-off. The inhibition cut-off value was set at $C_1 = 40.1$ for the HPPTP and 37.1 for the CDPK. The cervical smears were then tested for infection by *Chlamydia trachomatis*, using our assay including IR. If both the internal reference sample and the *Chlamydia* sample were negative, the sample was regarded as inhibited, so it was examined again (N = 6). If the sample turned out to be negative again, it was considered impossible to interpret (N = 3). The internal reference sample was not considered when the *Chlamydia* sample was found to be positive. Samples were regarded as negative if the *Chlamydia* was negative and the internal reference sample was positive. The originally positive and negative COBAS test results were confirmed.

40 Discussion

[0057] One of the characteristics that are important for the amplification of microorganisms in clinical samples is the monitoring and checking of the procedure with the aid of internal references. For a pathogen-specific real-time PCR assay, the ideal internal reference sample would have to be added to the clinical samples before the treatment, the recovery during the nuclein extraction and the replication either in a multiplex or in a competitive PCR with direct detection by fluorescent probes. None of the real-time PCR assays described so far⁷⁻⁹ has such an internal reference capable of checking the sample preparation and the inhibition in the step in which *Chlamydia trachomatis* is detected. Most other real-time assays use other microorganisms for monitoring the extraction of DNA, an example being the herpesvirus PhHV¹⁰. Although this method can monitor the efficiency of the DNA preparation, it cannot monitor the primary location-specific inhibition, and since these assays are mostly based on duplex PCR, it can be assumed that competition between the two amplicons leads to a reduced sensitivity of the assays. The differences between the HPPTP with only 200 cfu of internal reference sample in comparison with the 2000 cfu for the CDPK procedure is most probably the result of the fact that the HPPTP assay leads to pure DNA, while the CDPK only gives a preparation that is clean enough for the assay. It is interesting that no difference was found in the diagnostic sensitivity between the two procedures, most probably because, in a *Chlamydia*-positive epithelial cell, a single *Chlamydia* inclusion can contain more than 10,000 *Chlamydia* particles, each having about 10 plasmid targets. The internal reference represented in this study can monitor simultaneously the DNA preparation efficiency and the primer-specific PCR inhibition in the same PCR assay. This fact, in combination with the rapid and easier Roche COBAS CT DNA preparation kit, makes for a very reliable, rapid and

cost-effective real-time PCR *Chlamydia* assay that is highly suitable for routine work.

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[0058]

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Example 2

[0059] As described above, it was found that problems occur with the observation of *Chlamydia trachomatis* under certain conditions. An article by Söderblom et al. (*Euro Surveill.* 2006 Dec. 7; 11 (12): E061207.1) entitled "Impact of a genetic variant of *Chlamydia trachomatis* on national detection rates in Sweden" reports that part of the sexually transmitted *Chlamydia trachomatis* infections could not be observed in Sweden by standard laboratory tests produced by Abbott and Roche, and that *Chlamydia* bacteria with a variation in the genetic region at which the primers had been directed were not observed. Tests carried out by the method used in Example 1 have shown that such *Chlamydia trachomatis* types can indeed be observed by using primer pairs according to the present invention.

SEQUENCE LISTING

[0060]

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<110> Dutch Association for Christian Higher Education,
Scientific Research and Patient Care, Amsterdam, the Netherlands

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<120> Method for the detection of *Chlamydia trachomatis* and a kit for it

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<151> 2007-02-06

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50 **Claims**

1. Method for the detection of *Chlamydia trachomatis*, which method comprises the performance of a DNA amplification, using a primer pair, by using DNA that is derived from a sample as a template, and the detection of an amplification product, **characterized in that** the primer pair used for the DNA amplification is designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 4261 to 4320 and 4351 to 4391 in the nucleotide sequence of SEQ ID No. 1, so that the sequence in between these two regions can be amplified.
 55
 2. Method according to claim 1 , **characterized in that** the primer pair is designed on the basis of nucleotide sequences

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of the regions corresponding to the nucleotide numbers 4291 to 4320 and 4355- 4385 and preferably to the nucleotide numbers 4296 to 4318 and 4361 to 4382 of SEQ ID No. 1.

- 5 3. Method according to any one of the preceding claims, **characterized in that** the primer pair is designed on the basis of nucleotide sequences of the regions in SEQ ID No. 1 : GGATT GACTCCGACA ACGTATTC and TGCCCTT-TCT AATGGCAATGAT.
- 10 4. Kit for the detection of *Chlamydia trachomatis* by carrying out the PCR by using DNA obtained from a sample as a template, where the kit comprises a primer pair that is designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 4261 to 4320 and 4351 to 4391 in the nucleotide sequence of SEQ ID No. 1, so that the sequence in between these two regions can be amplified.
- 15 5. Kit according to claim 4, **characterized in that** the primer pair is designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 4261 to 4320 and 4351 to 4391 , preferably to the nucleotide numbers 4291 to 4320 and 4355-4385 and most preferably to the nucleotide numbers 4296 to 4318 and 4361 to 4382 of SEQ ID No. 1.
- 20 6. Kit according to claims 4-5, **characterized in that** the primer pair is designed on the basis of nucleotide sequences of the regions in SEQ ID No. 1 : GGATT GACTCCGACA ACGTATTC and TGCCCTTCT AATGGCAATGAT.
- 25 7. Primer pair designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 4261 to 4320 and 4351 to 4391 in the nucleotide sequence of SEQ ID No. 1, so that the sequence in between these two regions can be amplified.
- 30 8. Primer pair according to claim 7, **characterized in that** it is designed on the basis of nucleotide sequences of the regions corresponding to the nucleotide numbers 4261 to 4320 and 4351 to 4391 , preferably to nucleotide numbers 4291 to 4320 and 4355-4385 and most preferably to the nucleotide numbers 4296 to 4318 and 4361 to 4382 of SEQ ID No. 1.
- 35 9. Primer pair according to claims 7-8, **characterized in that** it is designed on the basis of nucleotide sequences of the regions in SEQ ID No. 1: GGATT GACTCCGACA ACGTATTC and TGCCCTTCT AATGGCAATGAT.
- 40 10. Primer pair according to claim 7, **characterized in that** the primers have the following sequences: 5'-GGA TTG ACT CCG ACA ACG TAT TC-3' (SEQ ID No. 5) and 5'-ATC ATT GCC ATT AGA AAG GGC A-3' (SEQ ID No. 6).
- 45 11. Primer set comprising a primer pair according to any one of claims 7-10, together with a supplementary primer that, as regards its sequence, differs from one of the primers of the primer pair in one nucleotide.
- 50 12. Primer set according to claim 11 , comprising the primer pair mentioned in claim 10 and a supplementary primer with the sequence 5'-GGA TTG ACT CCA ACA ACG TAT TC-3' (SEQ ID No. 7).
- 55 13. Hybridization probe comprising an oligonucleotide designed on the basis of the nucleotide sequence of a region corresponding to the nucleotide numbers 4320 to 4355 of SEQ ID No. 1.
- 60 14. Hybridization probe according to claim 13, with the sequence 5'-ACA CCG CTT TCT AAA CCG CCT ACA CGT AA-3' (Seq. ID. No. 8).

Patentansprüche

- 50 1. Verfahren zum Nachweis von *Chlamydia trachomatis*, wobei das Verfahren die Durchführung einer DNA-Amplifikation unter Verwendung eines Primerpaars unter Verwendung von DNA, die von einer Probe als Matrize abgeleitet ist, und den Nachweis eines Amplifikationsprodukts umfasst, **dadurch gekennzeichnet, dass** das zur DNA-Amplifikation verwendete Primerpaar aufgrund von Nucleotidsequenzen der Regionen entworfen ist, die den Nucleotidnummern 4261 bis 4320 und 4351 bis 4391 in der Nucleotidsequenz der SEQ ID Nr. 1 entsprechen, so dass die Sequenz zwischen diesen beiden Regionen amplifiziert werden kann.
- 55 2. Verfahren gemäß Anspruch 1, **dadurch gekennzeichnet, dass** das Primerpaar aufgrund von Nucleotidsequenzen

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der Regionen entworfen ist, die den Nucleotidnummern 4291 bis 4320 und 4355 - 4385 und vorzugsweise den Nucleotidnummern 4296 bis 4318 und 4361 bis 4382 der SEQ ID Nr. 1 entsprechen.

- 5 3. Verfahren gemäß einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** das Primerpaar aufgrund von Nucleotidsequenzen der Regionen in SEQ ID Nr. 1 entworfen ist: GGATT GACTCCGACA ACGTATTC und TGCCCTTTCT AATGGCAATGAT.
- 10 4. Kit zum Nachweis von *Chlamydia trachomatis* durch Ausführen der PCR unter Verwendung von DNA, die von einer Probe als Matrize erhalten wurde, wobei der Kit ein Primerpaar umfasst, das aufgrund von Nucleotidsequenzen der Regionen entworfen ist, die den Nucleotidnummern 4261 bis 4320 und 4351 bis 4391 in der Nucleotidsequenz der SEQ ID Nr. 1 entsprechen, so dass die Sequenz zwischen diesen beiden Regionen amplifiziert werden kann.
- 15 5. Kit gemäß Anspruch 4, **dadurch gekennzeichnet, dass** das Primerpaar aufgrund von Nucleotidsequenzen der Regionen entworfen ist, die den Nucleotidnummern 4261 bis 4320 und 4351 bis 4391, vorzugsweise den Nucleotidnummern 4291 bis 4320 und 4355 - 4385 und am stärksten bevorzugt den Nucleotidnummern 4296 bis 4318 und 4361 bis 4382 der SEQ ID Nr. 1 entsprechen.
- 20 6. Kit gemäß den Ansprüchen 4 - 5, **dadurch gekennzeichnet, dass** das Primerpaar aufgrund von Nucleotidsequenzen der Regionen in SEQ ID Nr. 1 entworfen ist: GGATT GACTCCGACA ACGTATTC und TGCCCTTTCT AATGGCAATGAT.
- 25 7. Primerpaar, das aufgrund von Nucleotidsequenzen der Regionen entworfen ist, die den Nucleotidnummern 4261 bis 4320 und 4351 bis 4391 in der Nucleotidsequenz der SEQ ID Nr. 1 entsprechen, so dass die Sequenz zwischen diesen beiden Regionen amplifiziert werden kann.
- 30 8. Primerpaar gemäß Anspruch 7, **dadurch gekennzeichnet, dass** es aufgrund von Nucleotidsequenzen der Regionen entworfen ist, die den Nucleotidnummern 4261 bis 4320 und 4351 bis 4391, vorzugsweise den Nucleotidnummern 4291 bis 4320 und 4355 - 4385 und am stärksten bevorzugt den Nucleotidnummern 4296 bis 4318 und 4361 bis 4382 der SEQ ID Nr. 1 entsprechen.
- 35 9. Primerpaar gemäß den Ansprüchen 7 - 8, **dadurch gekennzeichnet, dass** es aufgrund von Nucleotidsequenzen der Regionen in SEQ ID Nr. 1 entworfen ist: GGATT GACTCCGACA ACGTATTC und TGCCCTTTCT AATGGCAATGAT.
- 40 10. Primerpaar gemäß Anspruch 7, **dadurch gekennzeichnet, dass** die Primer die folgenden Sequenzen aufweisen: 5'-GGA TTG ACT CCG ACA ACG TAT TC-3' (SEQ ID Nr. 5) und 5'-ATC ATT GCC ATT AGA AAG GGC A-3' (SEQ ID Nr. 6).
- 45 11. Primerset, umfassend ein Primerpaar gemäß einem der Ansprüche 7 - 10, zusammen mit einem ergänzenden Primer, der sich in Bezug auf seine Sequenz von einem der Primer des Primerpaars in einem Nucleotid unterscheidet.
- 50 12. Primerset gemäß Anspruch 11, umfassend das in Anspruch 10 erwähnte Primerpaar und einen ergänzenden Primer mit der Sequenz 5'-GGA TTG ACT CCA ACA ACG TAT TC-3' (SEQ ID Nr. 7).
13. Hybridisierungssonde, umfassend ein Oligonucleotid, das aufgrund von der Nucleotidsequenz einer Region entworfen ist, die den Nucleotidnummern 4320 bis 4355 der SEQ ID Nr. 1 entspricht.
14. Hybridisierungssonde gemäß Anspruch 13, mit der Sequenz 5'-ACA CCG CTT TCT AAA CCG CCT ACA CGT AA-3' (SEQ ID Nr. 8).

Revendications

- 55 1. Procédé de détection de *Chlamydia trachomatis*, lequel procédé comprend la réalisation d'une amplification d'ADN, utilisant une paire d'amorces, en utilisant de l'ADN qui est dérivé d'un échantillon comme matrice, et la détection d'un produit amplifié, **caractérisé en ce que** la paire d'amorces utilisée pour l'amplification d'ADN est conçue sur la base des séquences nucléotidiques des régions correspondant aux nucléotides numéros 4261 à 4320 et 4351 à 4391 dans la séquence nucléotidique de SEQ ID NO : 1, de telle manière que la séquence entre ces deux régions

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peut être amplifiée.

2. Procédé selon la revendication 1, **caractérisé en ce que** la paire d'amorces est conçue sur la base des séquences nucléotidiques des régions correspondant aux nucléotides numéros 4291 à 4320 et 4355 à 4385 et de préférence aux nucléotides numéros 4296 à 4318 et 4361 à 4382 de SEQ ID NO : 1.
5
3. Procédé selon l'une quelconque des revendications précédentes, **caractérisé en ce que** la paire d'amorces est conçue sur la base des séquences nucléotidiques des régions dans SEQ ID NO : 1 : GGATT GACTCCGACA ACGTATTC et TGCCCTTTCT AATGGCAATGAT.
10
4. Kit pour la détection de *Chlamydia trachomatis* en réalisant la PCR en utilisant l'ADN obtenu d'un échantillon comme matrice, où le kit comprend une paire d'amorces qui est conçue sur la base des séquences nucléotidiques des régions correspondant aux nucléotides numéros 4261 à 4320 et 4351 à 4391 dans la séquence nucléotidique de SEQ ID NO : 1, de telle manière que la séquence entre ces deux régions peut être amplifiée.
15
5. Kit selon la revendication 4, **caractérisée en ce que** la paire d'amorces est conçue sur la base des séquences nucléotidiques des régions correspondant aux nucléotides numéros 4261 à 4320 et 4351 à 4391, de préférence aux nucléotides numéros 4291 à 4320 et 4355 à 4385 et de manière préférée entre toutes aux nucléotides numéros 4296 à 4318 et 4361 à 4382 de SEQ ID NO : 1.
20
6. Kit selon les revendications 4 et 5, **caractérisé en ce que** la paire d'amorces est conçue sur la base des séquences nucléotidiques des régions dans SEQ ID NO : 1 : GGATT GACTCCGACA ACGTATTC et TGCCCTTTCT AATGGCAATGAT.
25
7. Paire d'amorces conçue sur la base des séquences nucléotidiques des régions correspondant aux nucléotides numéros 4261 à 4320 et 4351 à 4391 dans la séquence nucléotidique de SEQ ID NO : 1, de telle manière que la séquence entre ces deux régions peut être amplifiée.
30
8. Paire d'amorces selon la revendication 7, **caractérisée en ce qu'elle** est conçue sur la base des séquences nucléotidiques des régions correspondant aux nucléotides numéros 4261 à 4320 et 4351 à 4391, de préférence aux nucléotides numéros 4291 à 4320 et 4355 à 4385 et de manière préférée entre toutes aux nucléotides numéros 4296 à 4318 et 4361 à 4382 de SEQ ID NO : 1.
35
9. Paire d'amorces selon les revendications 7 et 8, **caractérisée en ce qu'elle** est conçue sur la base des séquences nucléotidiques des régions dans SEQ ID NO : 1 : GGATT GACTCCGACA ACGTATTC et TGCCCTTTCT AATGGCAATGAT.
40
10. Paire d'amorces selon la revendication 7, **caractérisée en ce que** les amorces ont les séquences suivantes : 5'-GGA TTG ACT CCG ACA ACG TAT TC-3' (SEQ ID NO : 5) et 5'-ATC ATT GCC ATT AGA AAG GGC A-3' (SEQ ID NO : 6).
45
11. Ensemble d'amorces comprenant une paire d'amorces selon l'une quelconque des revendications 7 à 10, conjointement avec une amorce supplémentaire qui, en ce qui concerne sa séquence, diffère de l'une des amorces de la paire d'amorces dans un nucléotide.
50
12. Ensemble d'amorces selon la revendication 11, comprenant la paire d'amorces mentionnée dans la revendication 10 et une amorce supplémentaire avec la séquence 5'-GGA TTG ACT CCA ACA ACG TAT TC-3' (SEQ ID NO : 7).
55
13. Sonde d'hybridation comprenant un oligonucléotide conçu sur la base de la séquence nucléotidique d'une région correspondant aux nucléotides numéros 4320 à 4355 de SEQ ID NO : 1.
14. Sonde d'hybridation selon la revendication 13, avec la séquence 5'-ACA CCG CTT TCT AAA CCG CCT ACA CGT AA-3' (SEQ ID NO : 8).

Figure 1

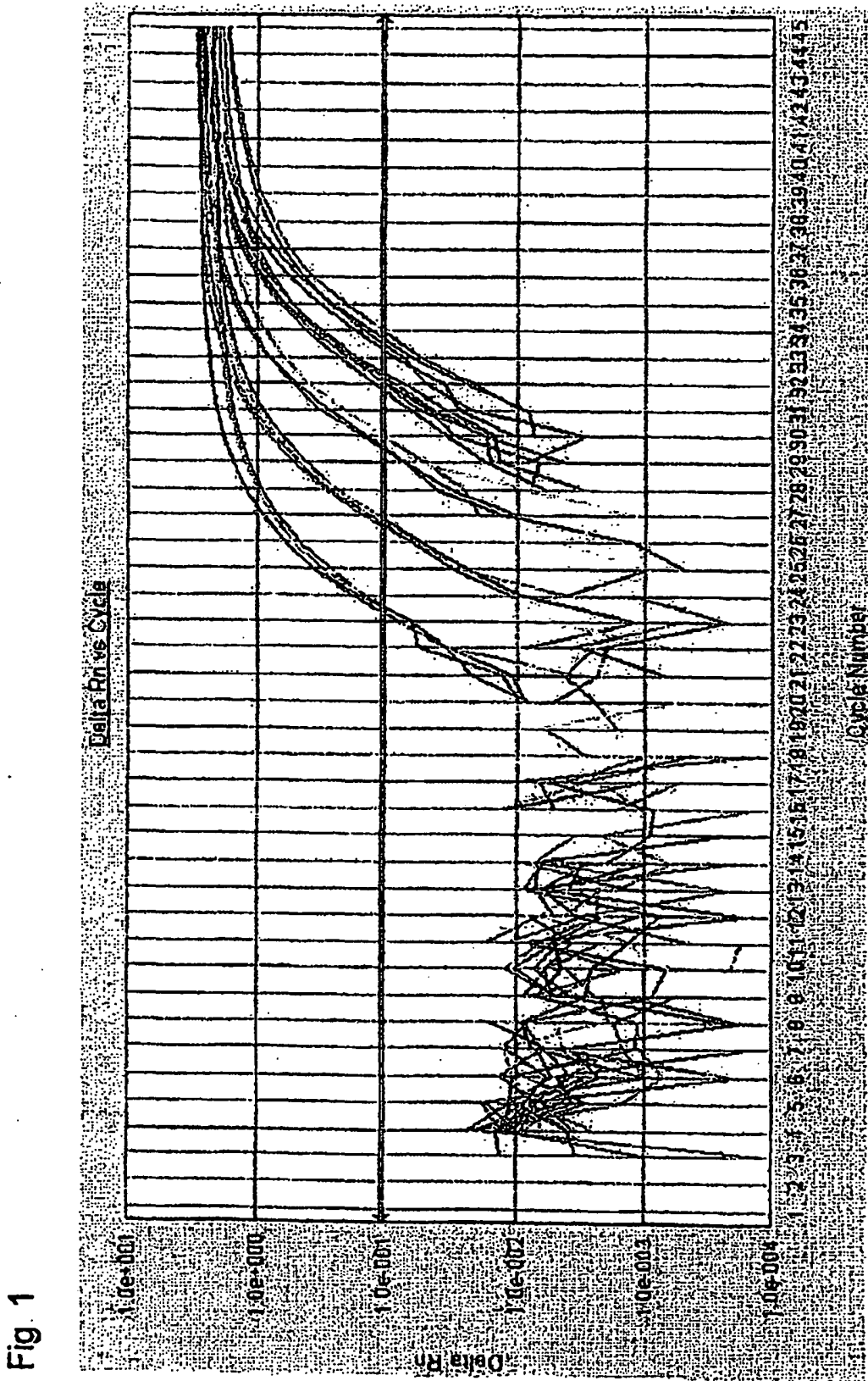
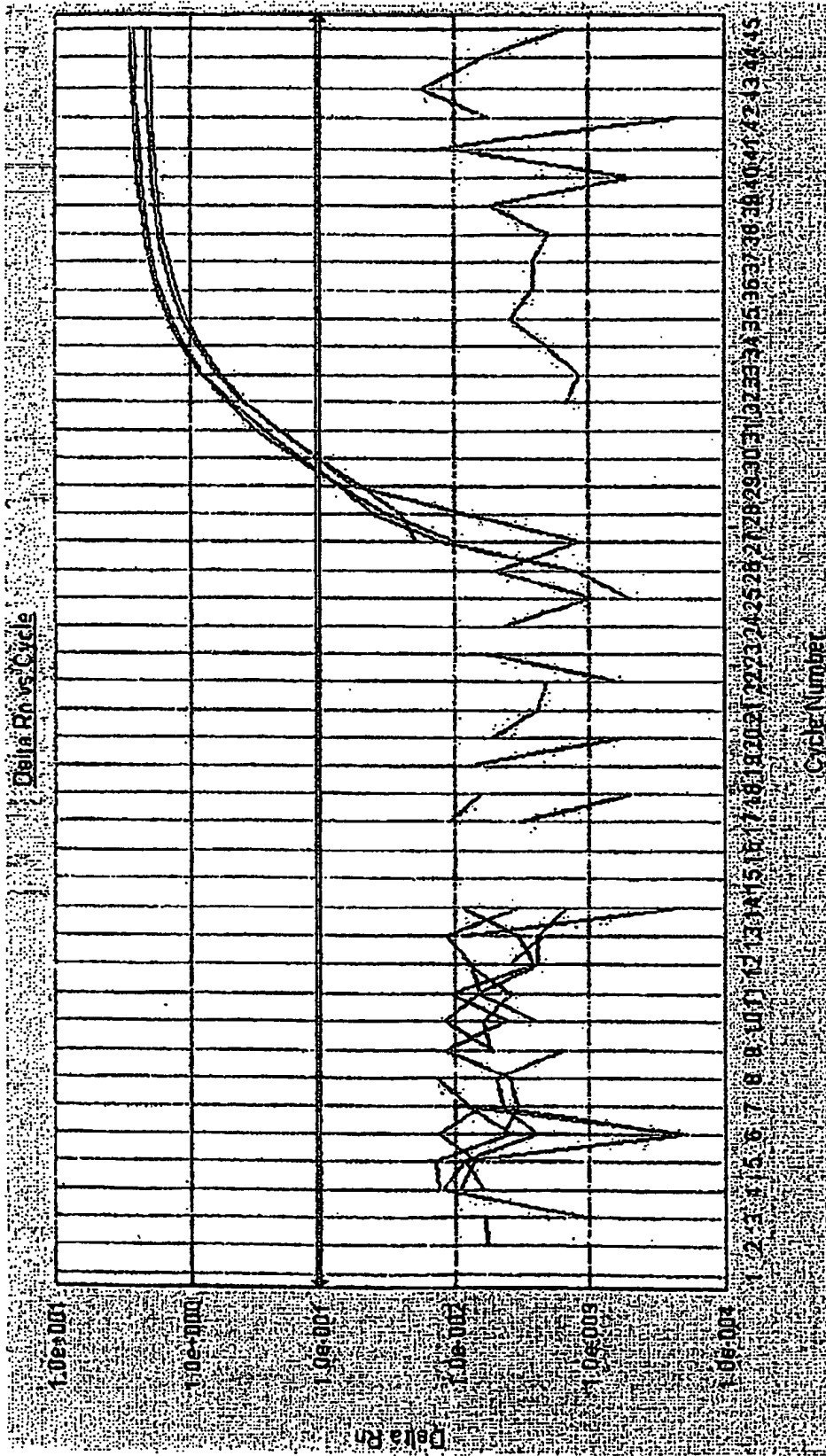


Fig. 1

Figure 2

Fig. 2



REFERENCES CITED IN THE DESCRIPTION

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